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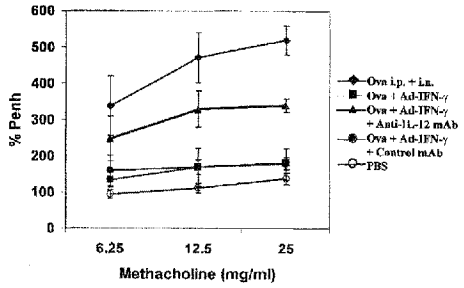
(54) Title: A METHOD FOR TREATING ALLERGIC DISEASE AND ASTHMA BY RECOMBINANT ADENOVIRUS-AND ADENO-ASSOCIATED VIRUS-MEDIATED IFN-Y GENE



(57) Abstract: The subject invention concerns an effective therapy for asthma, including allergic disease, using cytokine gene expression therapy. The subject invention further pertains to the use of adenovirus-mediated IFN-,γ(Ad-IFN-,γ) gene transfer to prevent or treat allergic disease and asthma, including associated conditions such as allergen-induced airway inflammation and airway hyperresponsiveness. The subject invention includes a method for effectively attenuating allergen-induced airway inflammation and airway hyperresponsiveness by administering to the respiratory tract Ad-IFN--γ, to affect IL-12 and STAT-4 levels. The subject invention also provides compositions for gene therapy for asthma by the transfer of IFN-γ

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DESCRIPTION

A METHOD OF TREATING ALLERGIC DISEASE AND ASTHMA BY RECOMBINANT ADENOVIRUS- AND ADENO-ASSOCIATED VIRUS- MEDIATED IFN-γ GENE TRANSFER

Cross-Reference to Related Application

[0001] This application claims the benefit of U.S. Provisional application Serial No. 60/360,841, filed March 1, 2002.

Field of the Invention

[0002] The subject invention pertains to the field of asthma and allergen treatment, more particularly to the use of adenovirus as a transfer vector to facilitate such treatment.

Background of the Invention

[0003] Allergic asthma is a chronic inflammatory disorder often characterized by airway inflammation and airway hyperreactivity (AHR). It is a leading cause of morbidity and mortality in children, adults, and the elderly. Current therapy for asthma includes treatment with bronchodilators, inhaled steroids, and leukotriene modifiers. Antigen specific immune therapy has also been used to desensitize patients to specific allergens; however, it can be ineffective for many allergic asthmatics sensitive to multiple antigens. Similarly, inhaled corticosteriods have severe adverse effects along with suppression of Th1 and Th2 cytokine responses. Also, even with currently available therapies, the incidence of asthma has continued to increase over the last two decades. Thus, an allergic asthmatic therapy is needed that induces long term effects against a broad array of antigens while providing fewer adverse effects.

[0004] Allergic asthma is caused by the disregulated production of cytokines secreted by allergen specific T-helper type 2 (Th2) cells. T helper type 1 (Th1) cells downregulate Th2 cells and Th2 pathology. As illustrated in Figure 1, both interferon (IFN)-γ, a pleiotropic T helper type 1 (Th1) cell cytokine, and interleukin (IL)-12 induce Th1 cells. IFN-γ is capable of activating macrophages and dendritic cells to induce production of IL-12. This, however, is dependent

upon the presence of IFN-γ, which provides a stimulatory signal to IL-12 (Tang, C. *et al.*, "Th type 1-stimulating activity of lung macrophages inhibits Th2-mediated allergic airway inflammation by an IFN-gamma-dependent mechanism," *J. Immunol.*, 166:1471-1481 (2001)), which in turn induces Th1 responsiveness. IL-12 binds with high affinity to receptors located on T cells and natural killer (NK) cells, causing activation of tyrosine kinases and nuclear translocation of signal transducer and activator of transcription (STAT) 4, to trigger the promoter regions for the IFN-γ gene (Hasko, G., and Szabo, C., "IL-12 as a therapeutic target for pharmacological modulation in immune-mediated and inflammatory diseases: regulation of T helper 1/T helper 2 responses," *Br. J. Pharmacol.*, 127:1295-1304 (1999)). Studies have shown that mice deficient in STAT4 produce reduced amounts of IFN- γ in response to IL-12 and have impaired Th1 activity, indicating that STAT 4 is essential in IL-12 responses (Thierfelder, W. E. *et al.*, "Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells," *Nature*, 382:171-174 (1996) and Kaplan, M. H., *et al.*, "Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice," *Nature*, 382:174-177 (1996)).

[0005] Recent developments in cytokine gene therapy suggest several advantages over current therapeutic approaches used to treat allergic asthma (Barnes, P.J. *et al.*, "Cytokine-directed therapies for asthma," *J. Allergy Clin. Immunol.*, 108:S72-76 (2001), Alvarez, D. *et al.*, "Cytokine therapeutics for asthma: an appraisal of current evidence and future prospects," *Curr. Pharm. Des.*, 7:1059-1081 (2001), Chung, F., "Anti-inflammatory cytokines in asthma and allergy: interleukin-10, interleukin-12, interferon-gamma," *Mediators Inflamm.*, 10:51-59 (2001), and Stirling, R. G, and Chung, K. F., "New immunological approaches and cytokine targets in asthma and allergy," *Eur. Respir. J.*, 16:1158-174 (2000)). For example, the administration of recombinant IL-12 or IFN-γ have demonstrated reduced airway inflammation and airway hyperresponsiveness in murine models of allergic asthma (Schwarze, J. *et al.*, "Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice," *J. Allergy Clin. Immunol.*, 102:86-93 (1998), Lack, G., *et al.*, "Nebulized but not parenteral IFN-gamma decreases IgE production and normalizes airways function in a murine model of allergen sensitization," *J. Immunol.*, 152:2546-2554 (1994), Gavett, S. H. *et al.*, "Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice," *J. Exp. Med.*, 182:1527-1536 (1995), and Sur, S. *et al.*, "Mucosal IL-12 inhibits airway reactivity to methacholine and respiratory failure in

murine asthma," *Exp. Lung Res.*, 26:477-489 (2000)). In one example, IL-12 was locally administered in the lung via systemic route (Cohen, J., "IL-12 deaths: explanation and a puzzle," *Science*, 270:908 (1995) and Mohapatra, S.S., "IL-12 possibilités," *Science*, 269:1499 (1995)). Unfortunately, exogenous recombinant cytokines have a short half-life *in vivo*, and systemic administration at high doses, especially of IL-12, may cause substantial adverse effects. In another example, plasmid DNAs (pDNA) encoding IFN-γ delivered to lung demonstrated effectiveness in modulating asthma in murine models (Li, X.M. *et al.*, "Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice," *J. Immunol.*, 157:3216-3219 (1996) and Dow, S.W. *et al.*, "Systemic and local interferon gamma gene delivery to the lungs for treatment of allergen-induced airway hyperresponsiveness in mice," *Hum. Gene Ther.*, 10:1905-1914 (1999)). The pDNA strategy is, however, limited by the poor transfectability of lung cells, especially of lymphocytes, and hence uncertainty in terms of amount and frequency of gene delivery. Moreover, intratracheal delivery of cationic lipid and pDNA complexes is limited in the amount of plasmid that can be delivered and also requires several administrations to obtain sufficient expression of the cDNA delivered.

[0006] Adenoviruses are non-enveloped particles of size 70 nm containing a linear double stranded DNA of approximately 36,000 base pairs. They are easily prepared with high titers and can infect a wide range of cells, including non-dividing cells. An important feature of adenovirus-mediated gene expression is the ability to control the magnitude of gene expression in a dose-dependent manner.

[0007] Adeno-associated viruses have a particle diameter of 20 nm. They may integrate with relatively low efficiency, but in non-dividing cells such as muscle and neurons, they are capable of inducing high-level, long-term expression in the absence of a virus-associated inflammatory or cellular immune response.

[0008] Recently, replication-deficient adenoviruses have been used as a vehicle for transient gene expression, which permits transgene expression in a dose-dependent manner. However, treatments for asthma using adenovirus-mediated, or adeno-associated virus-mediated, IFN-γ (Ad-IFN-γ) gene transfer have not been investigated.

[0009] All documents and publications cited herein are incorporated by reference in their entirety, to the extent not inconsistent with the explicit teachings set forth herein.

Brief Summary of the Invention

[0010] The subject invention provides compositions and methods for the administration of cytokine gene therapy to mammals. In a preferred embodiment, the subject invention provides an adenovirus- or adeno-associated virus-mediated IFN-γ (Ad-IFN-γ) gene expression therapy for to treat and/or prevent asthma, including allergic asthma, and its associated conditions/disorders in humans.

[0011] In accordance with the subject invention, Ad-IFN-γ gene therapy modulates established inflammation and airway hyperreactivity. In one embodiment, an adenovirus or adeno-associated virus comprises a gene encoding IFN-γ, and medicaments containing it (*i.e.*, a stabilizer), are useful for treating asthma, in particular allergic asthma. In another embodiment, Ad-IFN-γ is administered via a mucosal route, such as an intranasal, ophthalmic, or intratracheal route. In a related embodiment, Ad-IFN-γ is administered to the respiratory tract to effectively attenuate allergen-induced airway inflammation and AHR through an IL-12 and STAT-4 dependent mechanism.

[0012] In a further embodiment, intranasal delivery of Ad-IFN-γ elevates production of IFN-γ in the lung. With elevated IFN-γ production, the levels of Th2- cytokines, IL-4, IL-5, serum IgE, and eosinophilia are lowered in asthmatics exposed to allergens, such as methacholine. Using the treatment of the subject invention results in less epithelial damage, mucus plugging, and eosinophil infiltration in asthmatic lungs exposed to allergens.

[0013] The subject invention is designed to effectively attenuate established allergen-induced airway inflammation and AHR. Experimental data indicates that intranasal IFN-γ gene transfer significantly inhibits production of IL-4, IL-5, ovalbumin (OVA) specific serum IgE, airway inflammation and hyperactivity. These results demonstrate that these effects are mediated by the IL-12 and STAT-4 pathway.

Brief Description of the Drawings

[0014] Figure 1 is a schematic representation of the biological process involved in inducing Th1 response.

[0015] Figures 2(A) through (C) illustrate the cloning and expression of recombinant Ad-IFN-γ and Ad-LacZ.

[0016] Figures 3(A) through (D) are graphical illustrations demonstrating the effect of Ad-IFN-γ on Th2 cytokine response in murine bronchial lymph nodes.

[0017] Figure 4 is a graphical illustration of IL-12 mAb activity in reversing Ad-IFN-γ induced reduction in AHR and lung inflammation.

[0018] Figures 5(A) through (D) are photomicrographs of histologies of mice lungs treated with anti-IL-12 mAb.

[0019] Figures 6(A) through (D) are graphical illustrations showing the ability of Ad-IFN-γ to reverse established Th2 cytokine response in murine bronchial lymph nodes.

[0020] Figure 7 is a graphical illustration of the level of AHR in OVA-sensitized and challenged mice treated either with Ad-IFN-γ or Ad-LacZ.

[0021] Figures 8(A) through (C) are high-powered magnifications of bronchiole and peribronchial regions in OVA-sensitized and challenged mice.

[0022] Figures 8A-1, 8B-1, anc 8C-1 are further enlargements of regions shown in Figures 8(A) through (C).

[0023] Figures 9(A) through (D) are graphical illustrations showing the results of anti-IL-12 mAb treatment in Ad-IFN-γ induced Th2 cytokine levels.

[0024] Figure 10 illustrates the prevention of airway hyperresponsiveness with Ad-IFN-γ.

[0025] Figures 11(A) and (B) are graphical illustrations of the effect of Ad-IFN-γ on eosinophils and antigen specific serum IgE respectively.

[0026] Figures 12(A) through (D) are photomicrographs of mice lung histologies.

[0027] Figures 13(A) through (D) are graphical illustrations showing the ability of Ad-IFN-γ to affect OVA-induced AHR and Th2 cytokine production in $STAT4^{-/-}$ mice.

[0028] Figure 14 is a graphical illustration demonstrating that Ad-IFN-γ does not significantly affect OVA-induced AHR in $STAT4^{-/-}$ mice.

[0029] Figures 15(A) and (B) are photomicrographs of histologies of $STAT-4^{-/-}$ mice lungs treated with Ad-IFN-γ.

Detailed Disclosure of the Invention

[0030] The present invention provides a method for treating asthma, in particular allergic asthma. In a preferred embodiment, replication deficient adenovirus is used for IFN-γ gene overexpression in the lung. Advantageously, Ad-IFN-γ therapy provides: (1) the expression of

the transgene in a dose-dependent manner at a specific tissue for an extended period of time, and (2) any overexpression is, transient and avoids undesired side effects. According to the present invention, treatment of asthma can be tailored to the needs of individuals who differ in their level of IFN-γ production and responsiveness. Further, the present invention can be used to complement therapies involving IFN-α or IFN-β.

[0031] The present invention can effectively reduce the functional and immunologic abnormalities associated with allergen sensitization and challenge. Further, the present invention can reverse allergic asthma in mammals, including humans. The term "mammals," as defined herein, refers to any vertebrate, including human, bovine, equine, canine, feline, porcine, and ovine animals.

[0032] In a preferred embodiment, the present invention can prevent the development of allergen-induced inflammation and AHR in the respiratory tract. Further, the present invention can attenuate, or even reverse to normal, established allergen-induced airway inflammation and AHR in the respiratory tract.

[0033] In one embodiment, an adenovirus or adeno-associated virus comprises a gene encoding IFN-γ, and medicaments containing it (*i.e.*, a stabilizer), are used in treating asthma, in particular allergic asthma. In another embodiment, Ad-IFN-γ is administered to the respiratory tract to effectively attenuate allergen-induced airway inflammation and AHR. Using the treatment of the subject invention results in less epithelial damage, mucus plugging, and eosinophil infiltration in asthmatic lungs exposed to allergens.

[0034] In a further embodiment, Ad-IFN-γ is administered intranasally. Intranasal Ad-IFN-γ transfer results in increased expression of IFN-γ and IL-12. With elevated IFN-γ production, the levels of Th2- cytokines, IL-4, IL-5, ovalbumin (OVA) specific serum IgE, and eosinophilia are lowered in asthmatics exposed to allergens, such as methacholine.

[0035] The subject invention is designed to effectively attenuate established allergen-induced airway inflammation and AHR. Experimental data indicates that intranasal IFN-γ gene transfer significantly inhibits production of IL-4, IL-5, ovalbumin (OVA) specific serum IgE, airway inflammation and hyperactivity. These results demonstrate that these effects are mediated by the IL-12 and STAT-4 pathway.

[0036] Following is an example illustrating procedures for making and practicing the invention. This example should be construed to include obvious variations and is not limiting. Unless noted otherwise, all solvent mixture proportions are by volume and all percentages are by weight.

[0037] The methods traditionally used in molecular biology, such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in a cesium chloride gradient, agarose or acrylamide gel electrophoresis, purification of DNA fragments by electroelution, phenol or phenol/chloroform extraction of proteins, ethanol or isopropanol precipitation of DNA in a saline medium, transformation in Escherichia coli, and the like, are well known to a person skilled in the art and are amply described in the literature (Maniatis T. *et al.*, "Molecular Cloning, a Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., (1982); Ausubel, F.M. *et al.* (eds), "Current Protocols in Molecular Biology," John Wiley & Sons, New York, (1987)).

Example 1:

[0038] In this Example, the effectiveness of Ad-IFN-γ treatment for allergic asthma was assessed.

Materials

[0039] In this Example, a BALB/c mouse model of established allergic asthma was used to examine the impact of intranasal IFN-γ gene transfer on allergic inflammation immunopathology and airway hyperactivity. Female 6-8 weeks old wild type and $STAT4^{-/-}$ BALB/c mice from Jackson Laboratory (Bar Harbor, ME) were maintained in pathogen-free conditions.

Cloning and recombination of adenoviral vecotrs

[0040] Murine IFN-γ cDNA was cloned into adenoviral transfer vector pSHUTTLE-CMV (Stratagene, CA) at Kpril and Xhol sites. The left and right arms of pSHUTTLE-CMV vector contains Ad5 nucleotides 35,931-35,935 and 3,534-5,790, which mediate homologous recombination with pAdEasy-1 vector in *E.coli*, plus inverted terminal repeat (TTR) and packaging signal sequences (nucleotides 1-480 of Ad5) required for viral production in mammalian cells. PAdEasy-1 adenoviral plasmid (Stratagene, CA) contains all Ad5 sequences except nucleotides 1-3,533 (encompassing the E1 gene) and nucleotides 28,130-30,820 (encompassing E3).

[0041] For generation of recombinant adenovirus plasmid, pSHUTTLE-CMV-IFN-γ/LacZ plasmids were linearized with PmeI and co-transformed with pAdEasy-1 plasmid into recombination proficient BJ5183 cells. The recombination was confirmed by PacI digestion. The recombined clones were re-transformed in DH5α cells for large-scale plasmid purification.

Generation and purification of recombinant adenovirus

[0042] HEK293 cells, which produce the deleted E1 genes in trans, were transfected with 4μg of PacI digested recombinant adenovirus plasmid DNA with LIPOFECTIN (Life Technologies, MD). Cells were harvested 7-10 days post-transfection, resuspended in PBS and recombinant virus was collected by 3-4 freeze-thaw cycles. The recombinant virus expressing murine IFN-γ and LacZ were termed Ad-IFN-γ and Ad-LacZ, respectively. The viruses were amplified by infecting fresh HEK-293 cells. Viruses were further purified by CsCl banding, a process that is well known to those skilled in the art and is disclosed by Becker *et al.*, "Use of recombinant adenovirus for metabolic engineering of mammalian cells," *Methods Cell Biol.*, 43:161-189 (1994). The viral band was extracted and CsCl removed by passing through CENTRICON-100 columns (Millipore, MA). Viral plaque assay was performed following known protocols, such as those described by He *et al.*, "Simplified system for generating recombinant adenoviruses," *Proc. Nat'l. Acad. Sci. U.S.A.*, 95:2509-2514 (1998).

[0043] Referring now to Figures 2(A) through (C), cloning and expression of recombinant Ad-IFN-γ and Ad-LacZ are shown. Complete cDNA of murine IFN-γ and *E.coli* LacZ were cloned into the adenoviral shuttle vector and the recombinant adenoviruses were isolated from the rec+ *E.coli* and verified by restriction digestion, as illustrated in Figure 2A. The expression of the purified recombinant adenoviruses were monitored in HEK-293 cells 24h after infection. The supernatants from the infected cells were analyzed for IFN-γ expression by ELISA, as illustrated in Figure 2B. The results in Figure 2B demonstrate that HEK-293 cells infected with AD-IFN-γ, and not Ad-LacZ, produce 17.4 ng/ml of IFN-γ after 48 h of transfection. Time course expression of IFN-γ *in vivo* was examined in BALB/c mice. IFN-γ expression was measured by ELISA in broncho-alveolar lavage (BAL) fluid collected on days 1, 2, 5, 8 and 10 from mice (n=4) administered with Ad-IFN-γ or Ad-LacZ (control). IFN-γ expression peaked on day 1 in BAL fluid from Ad-IFN-γ administered mice, as illustrated in Figure 2C, and then slowly

declined over the next few days and reached basal level by day ten. In contrast, no IFN-γ was detected in the BAL from mice receiving the control virus, Ad-LacZ, or HEK-293 cells infected with Ad-LacZ. These results show that the infection by adenovirus itself does not induce expression of IFN-γ.

Treatment according to the subject invention: Prevention of AHR

[0044] BALB/c wild type or STAT4$^{-/-}$ mice were sensitized i.p. with ovalbumin (OVA) (50 μg) absorbed by 2 mg of aluminum potassium sulfate (alum) on days 1 and 15. On day 29, 1.0 x 10^8 PFU of Ad-IFN-γ or Ad-LacZ was administered i.n., followed by intranasal challenge with 50 μg of OVA on days 30, 31, and 32. For depletion of IL-12, group of BALB/c mice were intranasally administered with 200 μg of anti-IL-12 mAb or isotype matched control mAb in the mornings of days 30, 31, and 32 and challenged on the same days in the evenings with 50 μg of OVA. The day following last challenge, day 33, AHR was measured in conscious mice to increasing concentrations of methacholine. On day 34, mice were bled, and then sacrificed, bronchial lymph nodes and lungs were removed and single cell suspension of bronchial lymph node cells were prepared and cultured *in vitro* either in the presence of 100 μg/ml OVA or medium alone.

[0045] The results of the immunization and treatment strategy is schematically shown in Figures 3(A) through (D). To examine the effect of intranasal Ad-IFN-γ administration on the cytokine production in the lung, single cell suspensions of bronchial lymph nodes were cultured in medium alone or with 100 μg/ml OVA for 48h and the amounts of IL-4, IL-5, IFN-γ and IL-12 in the supernatant were quantified. Ad-IFN-γ treatment significantly decreased OVA-induced production of IL-4 ($p< 0.01$) and IL-5 ($p< 0.01$) and increased OVA-induced IFN-γ ($p< 0.01$) and IL-12 ($p<0.01$) in the culture supernatants, when compared to control group that did not receive Ad-IFN-γ or the group receiving Ad-LacZ. Groups of control mice, which were not OVA-sensitized but administered with Ad-IFN-γ and challenged showed significant expression of IFN-γ ($p< 0.01$) and IL-12 ($p< 0.01$), but neither IL-4 nor IL-5, compared to those treated with Ad-lacZ. None of these cytokines could be detected in the culture supernatants in absence of OVA stimulation. These results suggest that Ad-IFN-γ treatment promotes a Th1-like response and suppresses a Th2-like response.

Treatment according to the subject invention: Reversal of established AHR

[0046] Mice sensitized i.p. with 50 μg OVA on days 1 and 15, were intranasally challenged with 50 μg of OVA on days 29 and 30. On day 44, 1.0 x 10^8 PFU of Ad-IFN-γ or Ad-LacZ was administered i.n., followed by booster challenge i.n. with 50 μg of OVA i.n. on days 45, 46, and 47 and AHR was measured on day 48. Mice were bled and sacrificed on day 49.

[0047] As illustrated in Figure 4, OVA-sensitized and challenged mice that received Ad-IFN-γ and isotype matched control mAb showed significantly lower AHR ($p< 0.01$) when compared to the OVA-sensitized and challenged group. Intranasal administration of anti-IL-12 mAb significantly reversed the reduction in AHR conferred by Ad-IFN-γ. Mice receiving Ad-IFN-γ and anti-IL-12 mAb showed significantly higher AHR ($p< 0.05$) when compared to mice receiving Ad-IFN-γ alone or Ad-IFN-γ +control mAb. There was no significant difference between the OVA sensitized and challenged group and the group receiving Ad-IFN-γ and anti-IL-12 mAb.

[0048] To examine the effect of anti-IL-12 mAb on Ad-IFN-γ–induced lung histology in OVA-sensitized mice, lung sections from Ad-IFN-γ-treated mice administered with anti-IL-12 mAb or an isotype matched control antibody were compared. As illustrated in Figures 5(A) and (B), Ad-IFN-γ mediated protection from lung inflammation was also reversed in the group receiving anti-IL-12 mAb with the presence of increased infiltration of mononuclear cells and polymorphs in the interstitial and peri-broncho-vascular regions, whereas the group receiving the Ad-IFN-γ + isotype matched control mAb showed significantly reduced lung pathology (Figure 5(B)) similar to Ad-IFN-γ treated group (Figure 12(B)). Together these results show that the Ad-IFN-γ–induced reduction in AHR and lung pathology is predominantly dependent on IL-12 expression.

[0049] To determine whether Ad-IFN-γ can reverse the established Th2 response, mice were sensitized with OVA twice at an interval of 15 days and then challenged i.n. on days 29 and 30 and again on days 45-47. As illustrated in Figures 6(A) through (D), intranasal administration of Ad-IFN-γ to OVA sensitized and challenged mice significantly decreased OVA-induced production of IL-4 ($p< 0.01$) and IL-5 ($p< 0.01$) and increased OVA-induced IFN-γ ($p< 0.01$) and IL-12 ($p<0.01$) in the bronchial lymph node cells, when compared to those from OVA-sensitized

and challenged mice and the group receiving Ad-LacZ. These results show that Ad-IFN-γ treatment can reverse an established Th2 response.

[0050] To examine whether Ad-IFN-γ administration can reverse established AHR, mice were treated with Ad-IFN-γ after i.n. OVA challenge. As illustrated in Figure 7, mice receiving Ad-IFN-γ following OVA-sensitization and challenge showed significantly ($P<0.01$) lower AHR when compared to mice that did not receive Ad-IFN-γ or received Ad-LacZ. This demonstrates that Ad-IFN-γ administration not only inhibits development of AHR but also reverses established AHR in murine models. Treatment with Ad-IFN-γ to OVA-sensitized and challenged mice significantly reduced epithelial damage, infiltration of mononuclear cells and polymorphs in the interstitial and peri-broncho-vascular regions, as illustrated in Figure 8(B) and in the enlarged region in Figure 8B-1, when compared to OVA-sensitized and challenged mice, as illustrated in Figure 8(A) and in the enlarged region in Figure 8A-1, or mice treated with Ad-LacZ, as illustrated in Figure 8(C) and in the enlarged region in Figure 8C-1. Taken together, these results show that administration of Ad-IFN-γ significantly reverses AHR and lung inflammation associated with established allergic condition in OVA sensitized and challenged mice.

[0051] To investigate the mechanism of IFN-γ action in reducing allergic inflammation and AHR, mice (n=8) were administered i.n. anti-IL-12 mAb or isotype matched control mAb (n=8) for three consecutive days. As illustrated in Figures 9(A) through (D), the effect of the treatment of anti-IL-12 mAb on the T helper cell response was examined by analyzing the cytokine expression pattern in the bronchial lymph node cells cultured either in medium alone or induced with OVA. The group that received anti-IL-12 mAb produced, significantly higher levels of IL-4 and IL-5 compared to the group that received Ad-IFN-γ and the control mAb ($p< 0.01$) and the group that received Ad-IFN-γ alone ($p< 0.01$). The differences in the expression of IL-4 and IL-5 were not significant between OVA-sensitized and challenged mice and those receiving Ad-IFN-γ + anti-IL-12 mAb. These results show that blocking IL-12 by anti-IL-12 mAb significantly blocks Ad-IFN-γ effect.

Treatment according to the subject invention: Measurement of Airway Hyperresponsiveness

[0052] Airway hyperresponsiveness to inhaled methacholine was measured using the whole body PLETHYSMOGRAPH (Buxco, Troy, N.Y.), using known procedures such as those disclosed by

Matsuse *et al.*, "Recurrent respiratory syncytial virus infections in allergen-sensitzed mice lead to persistent airway inflammation and hyperresponsiveness," *J. Immunol.*, 164:6483-6492 (2000).

[0053] To examine the effect of Ad-IFN-γ administration on allergic inflammation and asthma, the AHR, lung eosinophilia and IgE was measured in mice. Groups (n=6) of mice immunized i.p. twice with 50 μg/ml of OVA, were treated with either Ad-LacZ (n=6) or Ad-IFN-γ (n=8) and then challenged i.n. with OVA on days 30, 31 and 32. Their AHR was measured by whole-body plethysmography and compared with PBS as baseline. Both groups of control mice, those which received Ad-LacZ virus i.n. on day 29 prior to OVA challenge, and those which were OVA sensitized and challenged without any treatment, exhibited significant airway hyperresponsiveness to increasing concentrations of methacholine, as illustrated in Figure 10. No significant difference was observed between Ad-LacZ treated and untreated mice. In marked contrast, administration of Ad-IFN-γ i.n. prior to OVA challenge significantly reduced ($p<0.01$) the development of AHR compared to the control groups. There was no significant difference between Ad-IFN-γ treated mice and those treated with Ad-LacZ or Ad-IFN-γ without OVA sensitization. This effect was IFN-γ-specific, as the mice receiving Ad-LacZ did not significantly reduce AHR.

[0054] As illustrated in Figures 11(A) and (B), treatment with Ad-IFN-γ reduced the number of eosinophils in the BALF from 3.65×10^3 to 0.863×10^3 ($p<0.01$) when compared to the OVA-sensitized and challenged group or the group receiving Ad-LacZ, indicating that the reduction in eosinophils is specific to IFN-γ expression. Ad-IFN-γ administration also significantly reduced ($p<0.05$) OVA-specific serum IgE levels when compared to the OVA-sensitized and challenged group and the group receiving Ad-LacZ. These results show that Ad-IFN-γ treatment significantly reduces AHR, lung eosinophilia and allergen specific IgE.

[0055] As illustrated in Figures 12(A) through (D), lung inflammation was examined from formalin fixed, hematoxylin and eosin (H & E) stained lung sections from all treatment groups. The Ad-IFN-γ treated group exhibited reduced epithelial damage, less infiltration of mononuclear cells and polymorphs in the interstitial and peri-broncho-vascular regions (Figure 12(B)) compared to the control group, which either received Ad-LacZ (Figure 12(C)) or the OVA-sensitized and -challenged group (Figure 12(A)). Mice treated with Ad-IFN-γ did not show large inflammatory regions in the (Figures 12(B) and (D)) mice, however, very few inflammatory cells

appear around the peri-broncho vascular region in Ad-IFN-γ treated mice as seen in Figure 12(B). The group receiving Ad-IFN-γ and i.n. OVA alone (not OVA i.p.) had nearly normal lung morphology but with thickened epithelial layer (Figure 6D). A similar result was obtained with the group receiving Ad-LacZ and OVA i.n. (but not OVA i.p.). Thus, the recombinant adenovirus itself did not induce lung pathology.

Treatment according to the subject invention: Bronchial lymph node culture and assay for cytokines

[0056] Single cell suspensions of bronchial lymph nodes (3 x 10^6 cells/well of 24 well plate) were restimulated *in vitro* in the presence or absence of 100 μg/ml OVA. Supernatants were collected after 48h for cytokine ELISAs. ELISA for IL-4, IL-5, IL-12 and IFN-γ were performed using kits from R & D SYSTEMS (Minneapolis, MN) following manufacturer's protocol.

Treatment according to the subject invention: OVA-specific IgE analysis

[0057] To determine the OVA-specific IgE, microtitre plate was coated overnight at 4° C with 100 μl of OVA (5 μg/ml). Non-specific sites were blocked following three washes with PBST (0.5% Tween-20 in PBS). Mouse sera were incubated to the antigen-coated wells and bound IgE was detected with biotinylated anti-mouse IgE (02112D; Pharmingen, CA). Biotin anti-mouse IgE (021222D) reacts specifically with the mouse IgE of Igh^a and Igh^b haplotypes and does not react with other IgG isotypes. Diluted streptavidin-peroxidase conjugate was added, the bound enzyme detected with TMB, and the absorbance read at 450nm.

Treatment according to the subject invention: Lung histologyMice were sacrificed within 24 hours after the last challenge, and lung sections were subjected to paraffin embedding. Lung inflammation was assessed after the sections were stained with hematoxylin and eosin.The results of the study described in Example 1 show the following:

[0060] 1. Intranasal delivery of recombinant adenovirus expressing IFN-γ expresses high level IFN-γ in murine lung without causing any significant inflammatory response;

[0061] 2. Ad-IFN-γ significantly reduced OVA-induced airway hyperresponsiveness;

[0062] 3. Ad-IFN-γ promotes Th1 cytokine production and reduces Th2 cytokine production in thoracic lymph nodes;

[0063] 4. Ad-IFN-γ decreases the levels antigen-specific serum IgE antibodies and number of eosinophils bronchoalveolar lavage fluid;

[0064] 5. Ad-IFN-γ restores normal lung histology in OVA -sensitized and -challenged mice;

[0065] 6. IFN-γ mediates its protective anti-allergic response via the expression of IL-12, as concomitant administration of anti-IL-12 mAb significantly reverses the protective response of IFN-γ; and

[0066] 7. IFN-γ mediated anti-allergic response is dependent on the expression of STAT-4.

[0067] To further confirm the requirement of IL-12 in mediating the anti-inflammatory response of Ad-IFN-γ, the study was extended to STAT-$4^{+/+}$ and STAT-$4^{-/-}$ mice. Mice were treated with Ad-IFN-γ (n=8). Examination of the cytokine profiles from the bronchial lymph node cultures of $STAT4^{-/-}$ mice upon stimulation *in vitro* with OVA showed a significantly elevated expression of IL-12 ($p< 0.05$) and IFN-γ ($p< 0.01$), but no significant reduction in Th2 cytokines (IL-4 and IL-5) in mice receiving Ad-IFN-γ, when compared to OVA-sensitized and challenged mice, as illustrated in Figures 13(A) through (D). There was no significant difference in AHR between the group administered with Ad-IFN-γ and OVA-sensitized and challenged $STAT4^{-/-}$ group, as illustrated in Figure 14, even though a moderate decrease in AHR was observed in mice receiving Ad-IFN-γ. An analysis of lung sections revealed that both the OVA sensitized and challenged group and the group receiving Ad-IFN-γ showed similar lung pathology with increased infiltration of mononuclear cells in the peri-broncho-vascular region (Figures 15(A) and (B)). Similarly no significant difference was observed in OVA specific IgE levels between the two groups. These data indicate that the reduction in AHR, Th2 cytokines, and lung inflammation conferred by Ad-IFN-γ is predominantly STAT-4 dependent.

[0068] The present invention provides a method for enhancing the expression of IFN-γ in humans. As is known to the skilled artisan, the T helper cell differentiation pathway functions similarly in both mice and humans. Current data regarding certain gene therapies in immune responses suggest that results shown in murine models can analogously and successfully be induced in humans (Payne LG, Fuller DH, Haynes JR., "Particle-mediated DNA vaccination of mice, monkeys and men: looking beyond the dogma," *Curr Opin Mol Ther*, (5):459-66 (2002)). Moreover, IFN-γ has demonstrated an ability to decrease (i) IL-13- induced goblet cell hyperplasia and eosinophilia by diminished IL-13 signaling through upregulation of the IL-

13Rα2 decoy receptor (Ford *et al.*, 2001; Daines *et al.*, 2002); (ii) LTC4 production in murine and human macrophages (Boraschi *et al.*, 1987; Thivierge *et al.*, 2001), in human peripheral blood lymphocytes after wasp venom immunotherapy (Pierkes *et al.*, 1999), and in leukocytes of pollinosis patients (Krasnowska *et al.*, 2000); and (iii) TGF-β, and procollagen-I and -III, which cause fibrosis and airway remodeling (Gurujeyalakshmi *et al.*, 1995; Minshall *et al.*, 1997). This invention can be used to enhance IFN-γ expression both prophylactically and/or therapeutically to treat allergic diseases, including allergic asthma.

[0069] Inasmuch as the preceding disclosure presents the best mode devised by the inventor for practicing the invention and is intended to enable one skilled in the pertinent art to carry it out, it is apparent that methods incorporating modifications and variations will be obvious to those skilled in the art. As such, it should not be construed to be limited thereby but should include such aforementioned obvious variations.

Claims

We claim:

1. A method of enhancing IFN-γ expression to regulate the production of cytokines secreted by Th2 cells comprising administering an adenovirus comprising an IFN-γ gene in a mammal.

2. The method according to claim 1, wherein the adenovirus comprising the IFN-γ gene is administered to humans.

3. The method according to claim 1, wherein the adenovirus is administered to cells of the respiratory tract.

4. The method according to claim 1, wherein the adenovirus is in a medicament.

5. A method if enhancing IFN-γ expression to regulate the production of cytokines secreted by Th2 cells comprising administering an adeno-associated virus comprising an IFN-γ gene in a mammal.

6. The method according to claim 5, wherein the adenovirus comprising the IFN-γ gene is administered to humans.

7. The method according to claim 5, wherein the adenovirus is administered to cells of the respiratory tract.

8. The method according to claim 5, wherein the adenovirus is in a medicament.

9. A pharmaceutical composition comprising an adenovirus comprising an IFN-γ gene.

10. A pharmaceutical composition comprising an adeno-associated virus comprising an IFN-γ gene.

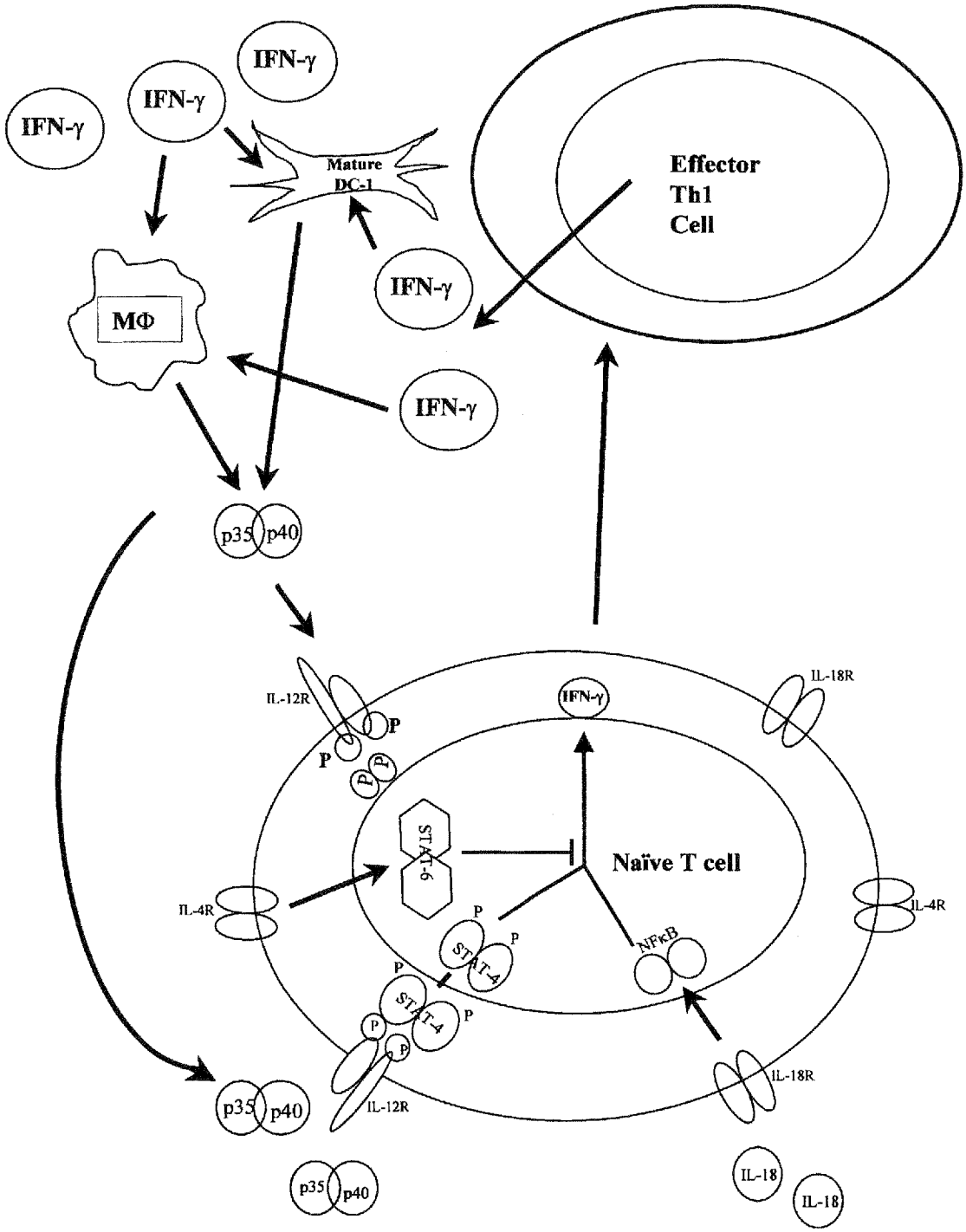
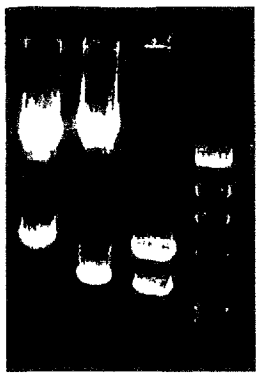

IFN-γ
IFN-γ
IFN-γ
Mature DC-1
Effector Th1 Cell
IFN-γ
MΦ
IFN-γ
p35
p40
IL-12R
P
IFN-γ
IL-18R
STAT-6
Naïve T cell
IL-4R
IL-4R
STAT-4
NFκB
STAT-4
IL-18R
IL-12R
p35
p40
p35
p40
IL-18
IL-18

FIG. 1

FIG. 2A



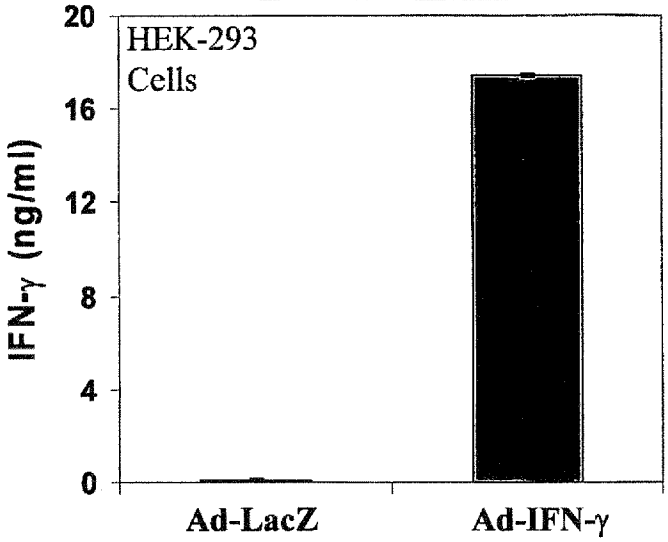


FIG. 2B

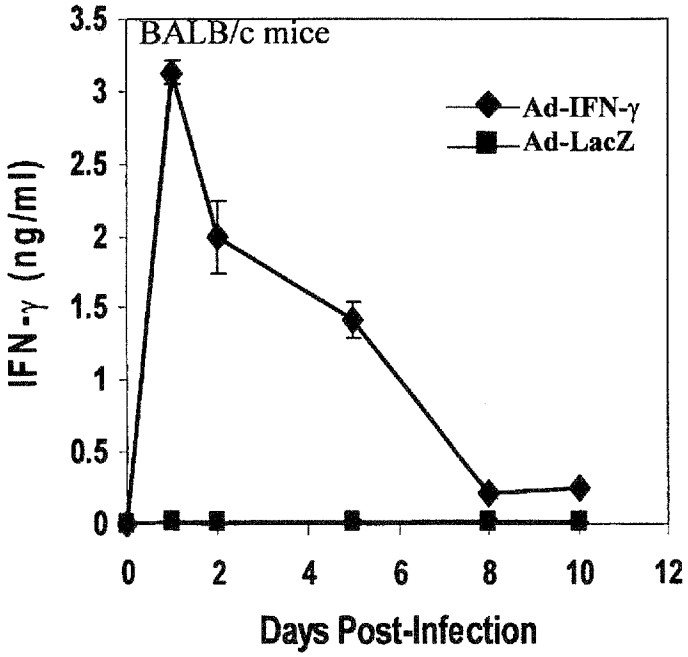


FIG. 2C

FIG. 3A

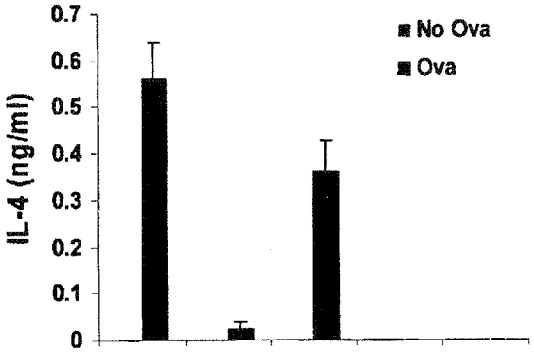


FIG. 3B

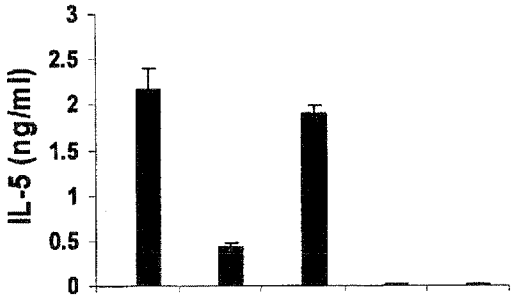


FIG. 3C

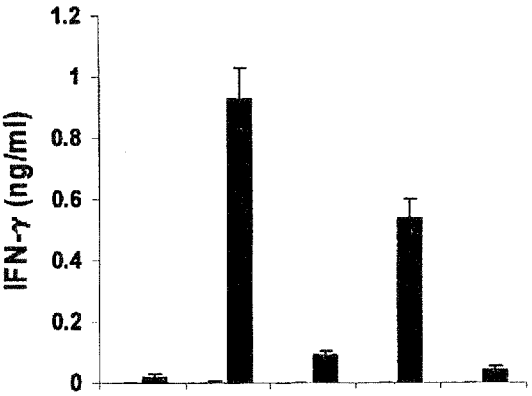
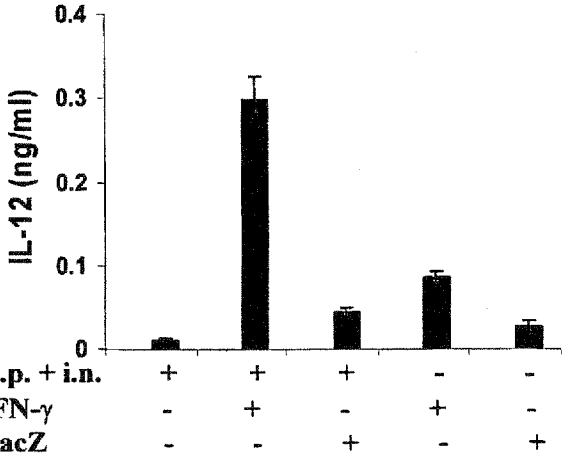


FIG. 3D



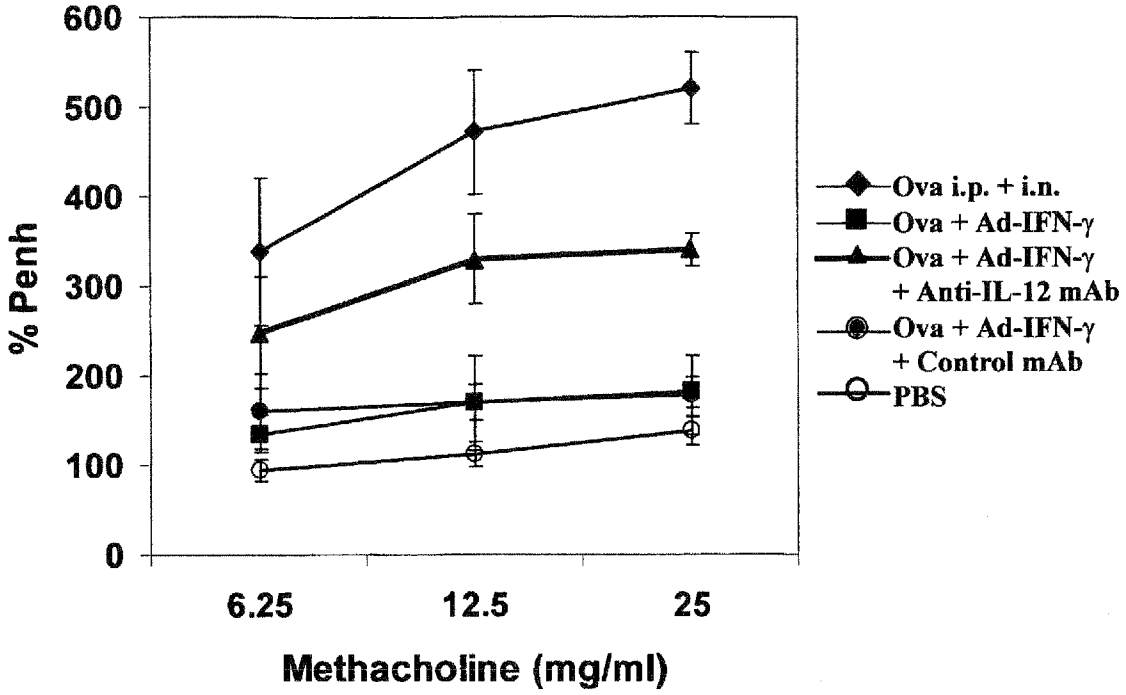


FIG. 4

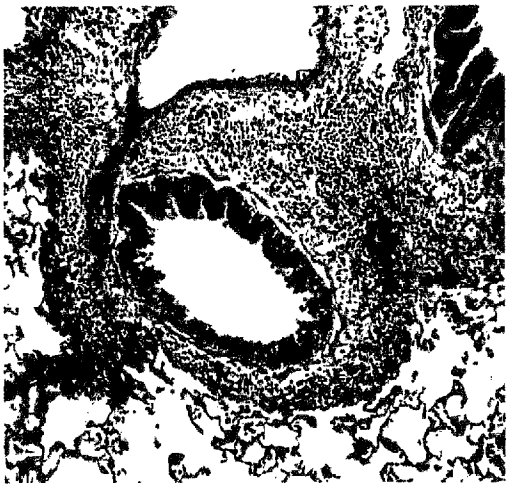


FIG. 5A

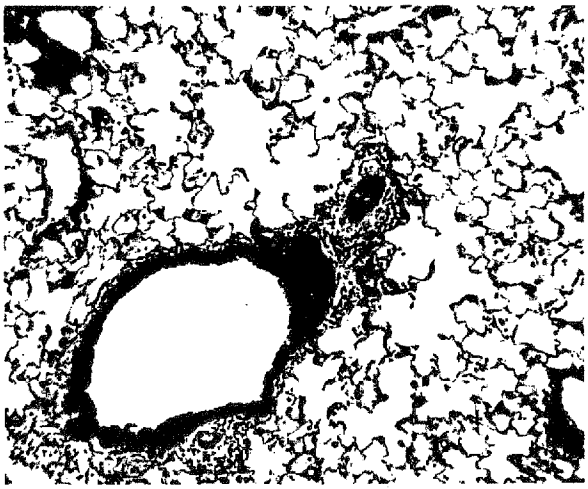


FIG. 5B

FIG. 6A

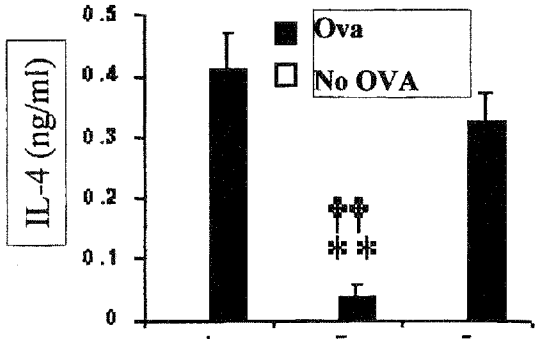


FIG. 6B

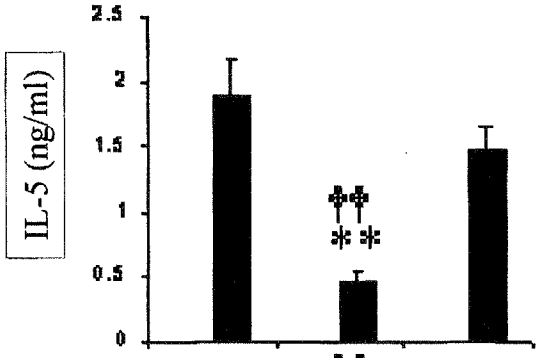


FIG. 6C

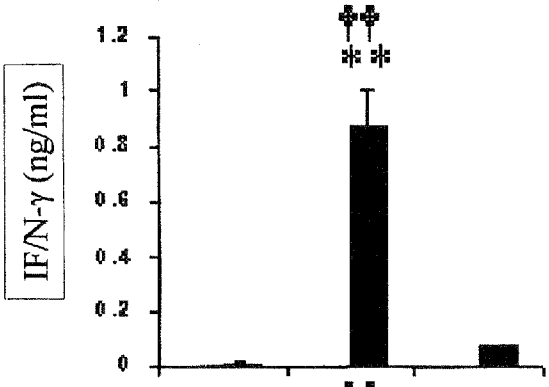


FIG. 6D

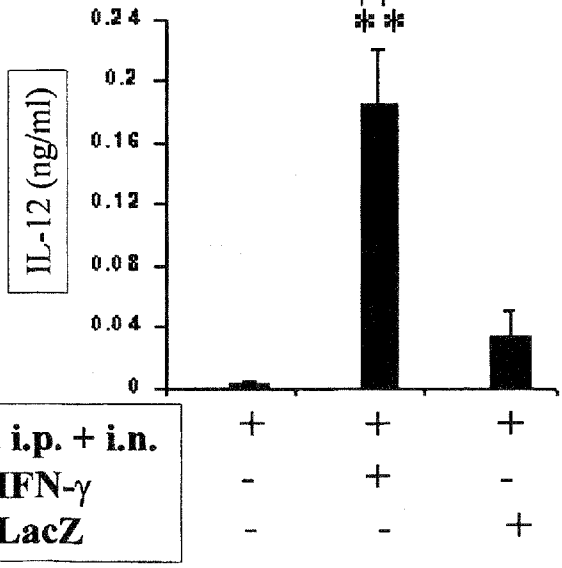


FIG. 7

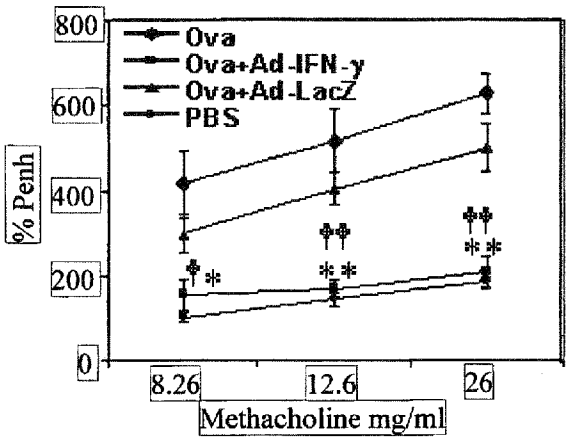


FIG. 8A

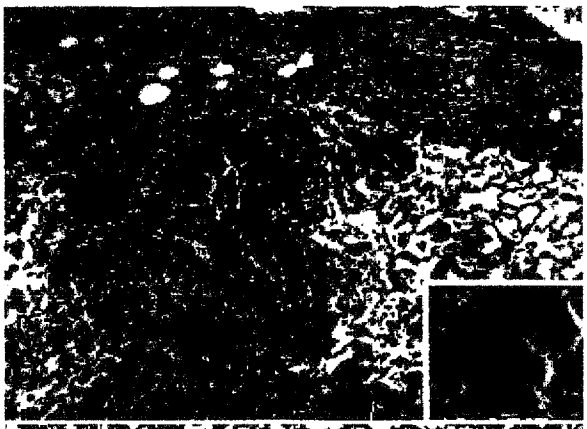


FIG. 8A-1

FIG. 8B

FIG. 8B-1

FIG. 8C

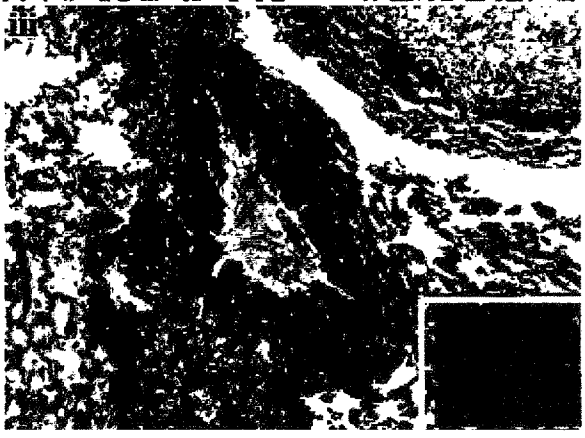


FIG. 8C-1

FIG. 9A

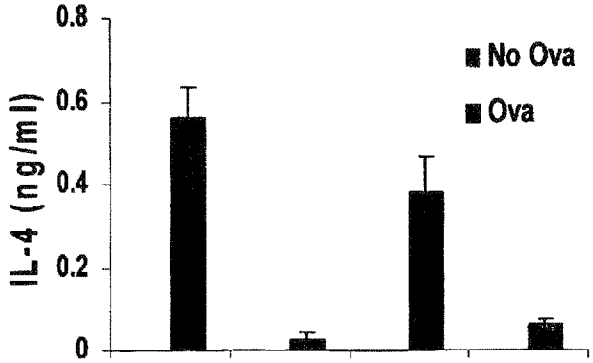


FIG. 9B

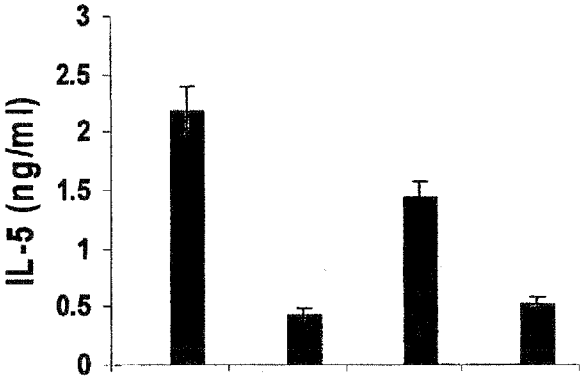


FIG. 9C

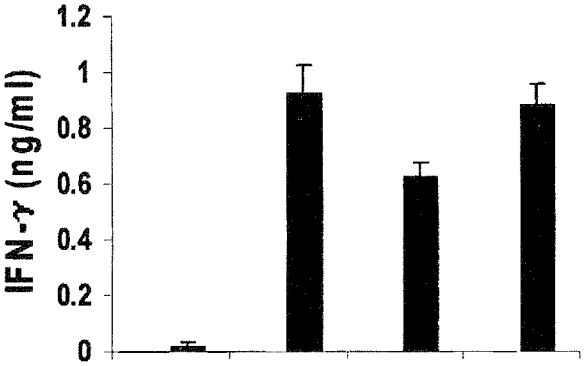
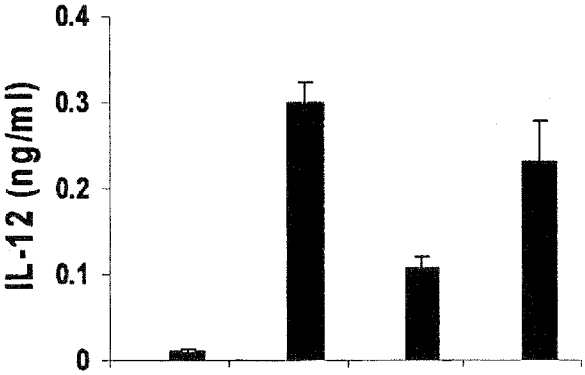


FIG. 9D



Ova i.p.+ i.n.	+	+	+	+
Ad-IFN-γ	-	+	+	+
Anti-IL-12 mAb	-	-	+	-
Control mAb	-	-	-	+

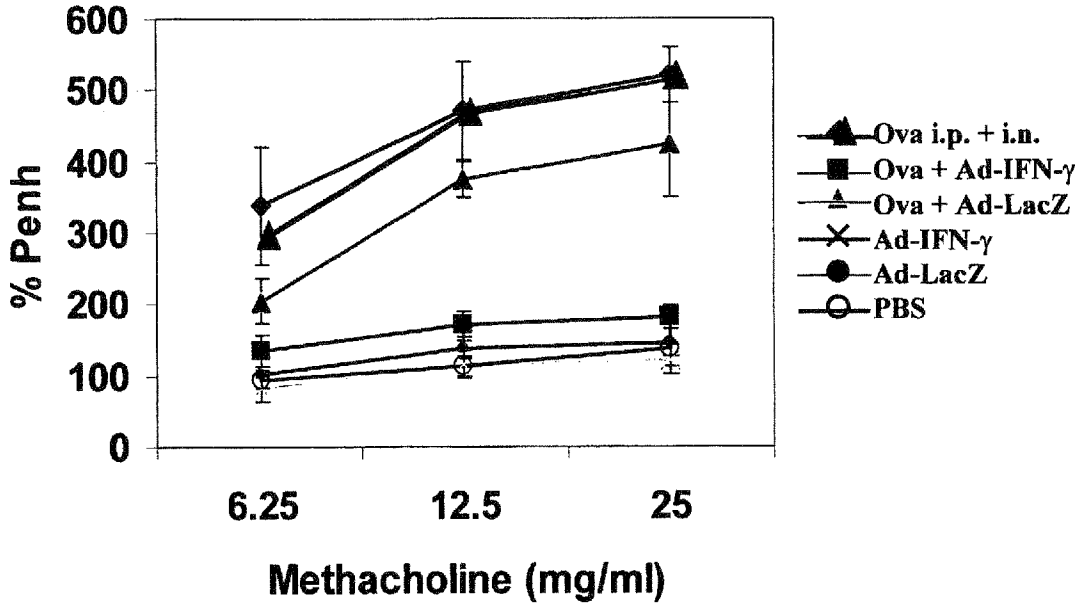


FIG. 10

FIG.11A

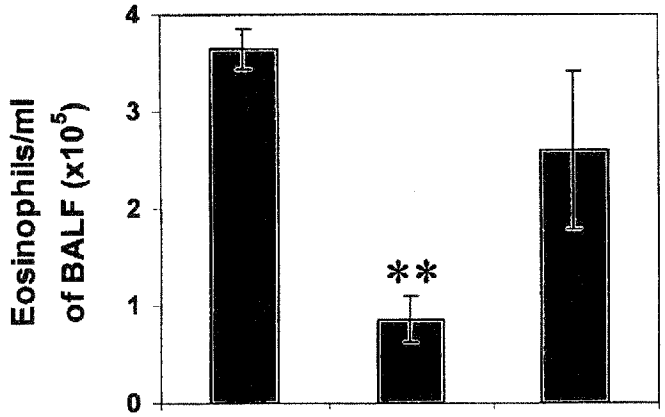
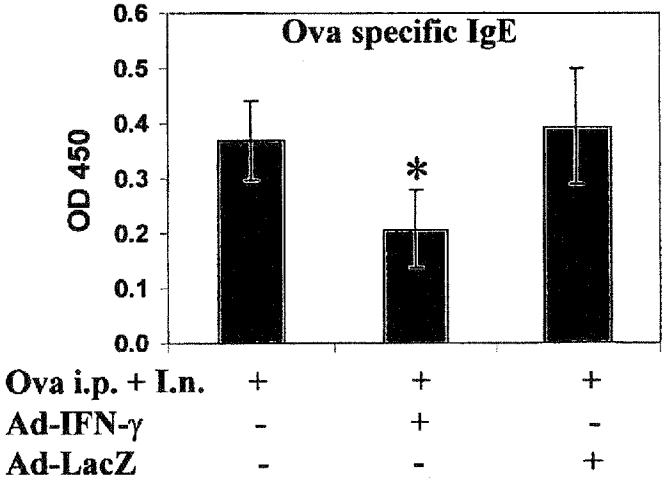


FIG. 11B



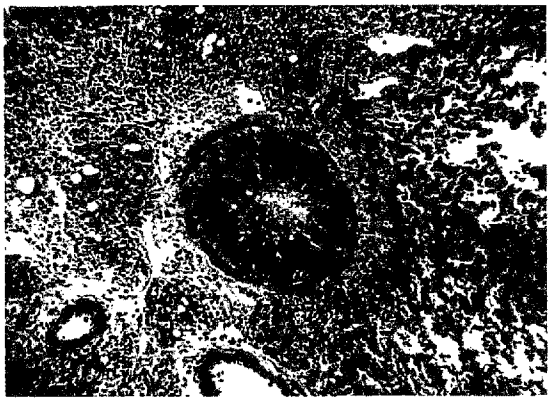


FIG. 12A

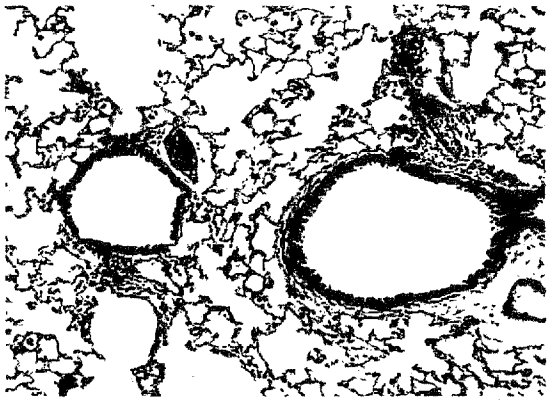


FIG. 12B

FIG. 12C

FIG. 12D

FIG. 13A

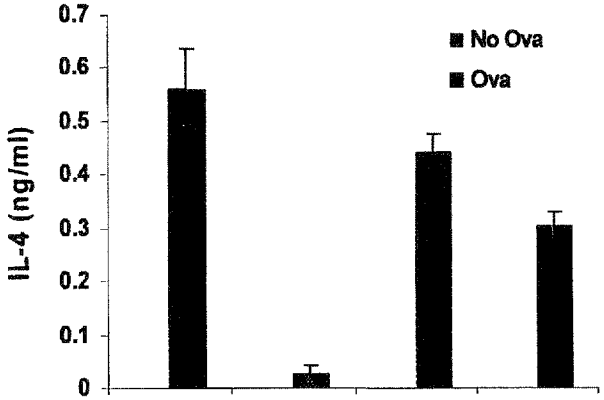


FIG. 13B

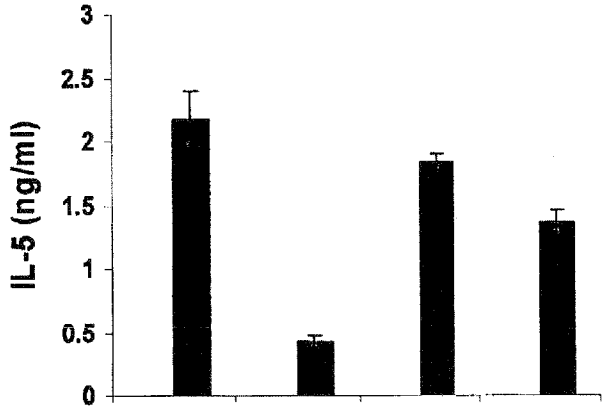


FIG. 13C

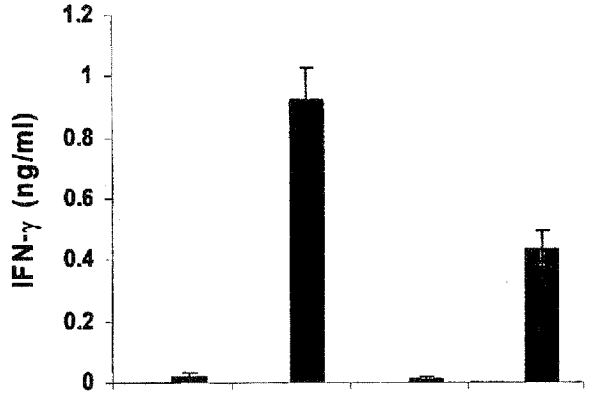
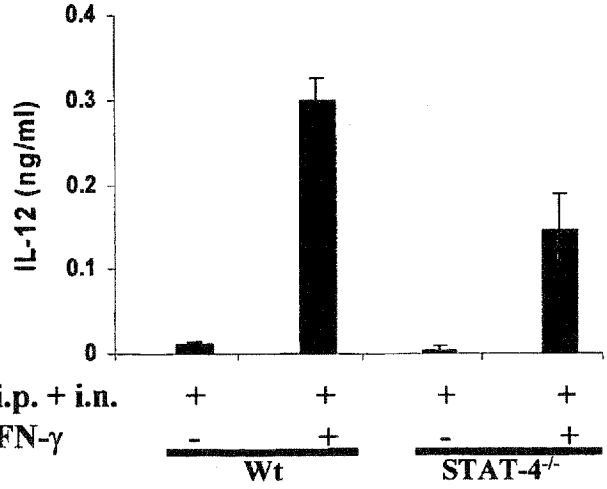


FIG. 13D



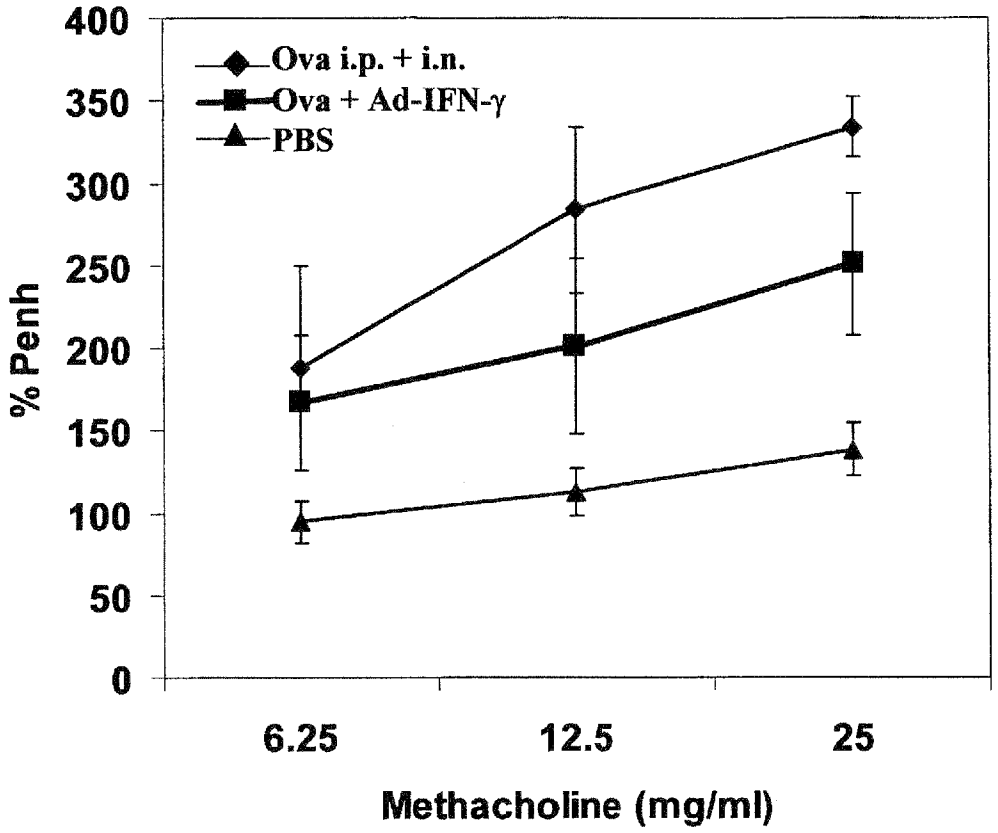

400
350
300
250
200
150
100
50
0
% Penh
Ova i.p. + i.n.
Ova + Ad-IFN-γ
PBS
6.25
12.5
25
Methacholine (mg/ml)

FIG. 14

FIG. 15A

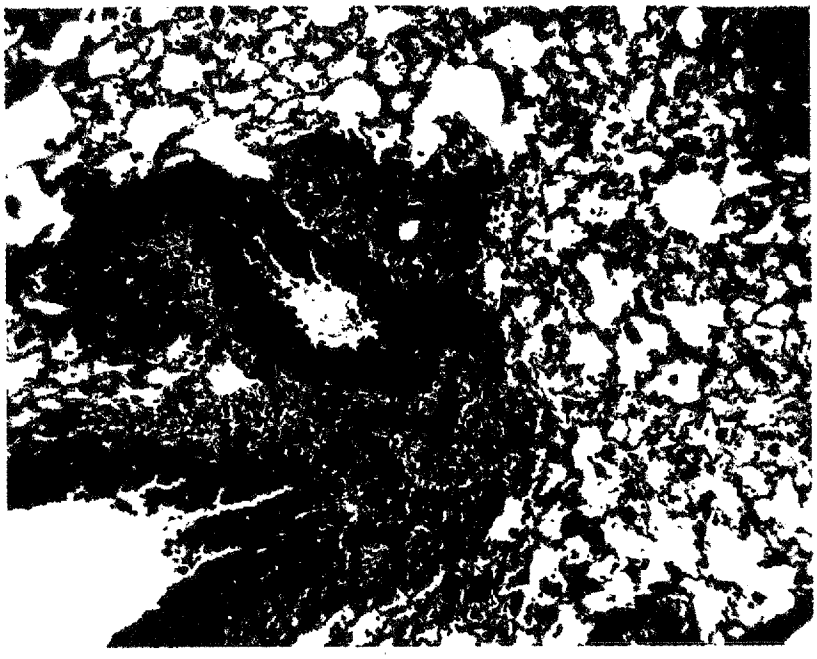
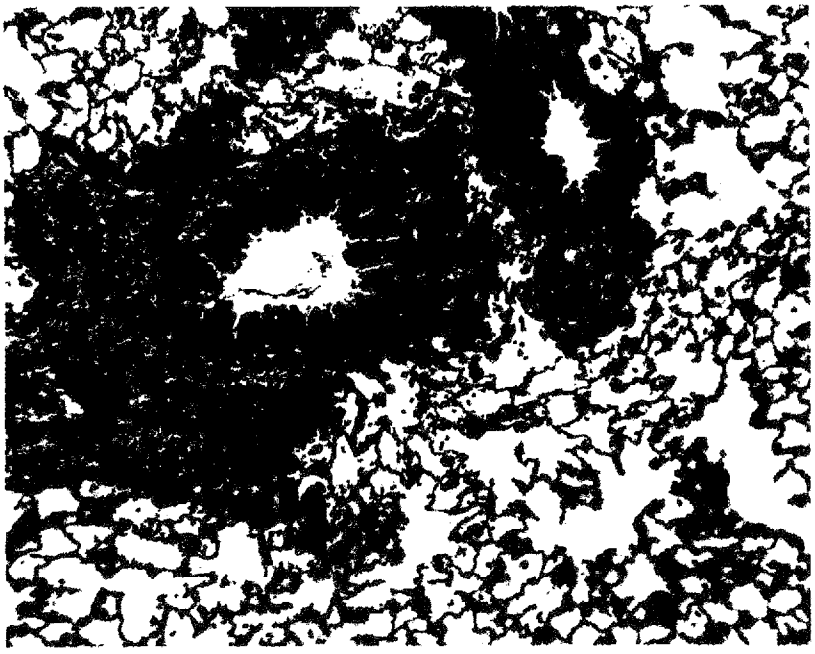


FIG. 15B



INTERNATIONAL SEARCH REPORT

In nal Application No
PCT/US 03/06535

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/57 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WALTER D M ET AL: "IL-18 gene transfer by adenovirus (ADV) into the lungs prevents and reverses allergen-induced airway hyperreactivity (AHR)." FASEB JOURNAL, vol. 14, no. 6, 20 April 2000 (2000-04-20), page A1066 XP002241813 Joint Annual Meeting of the American Association of Immunologists and the Clinical Immunology Society;Seattle, Washington, USA; May 12-16, 2000 ISSN: 0892-6638 page 6392, right-hand column, line 2 - line 3 page 6393, left-hand column, paragraph 1 - paragraph 3 --- -/--	1-4,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 May 2003	04/06/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nichogiannopoulou, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In ›nal Application No
PCT/US 03/06535

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RENER ET AL: "Construction and identification of a recombinant adenovirus which expresses human interferon-gamma" CHINESE JOURNAL OF BIOTECHNOLOGY, ALLERTON PRESS, NEW YORK, NY, US, vol. 13, no. 1, January 1997 (1997-01), pages 1-8, XP002111196 ISSN: 1042-749X page 7, line 5 - line 14	1-4,9
Y	US 6 218 180 B1 (COLOSI PETER C ET AL) 17 April 2001 (2001-04-17) claims 1,6,8	5-8,10
Y	LI X-M ET AL: "MUCOSAL IFN-GAMMA GENE TRANSFER INHIBITS, PULMONARY ALLERGIC RESPONSES IN MICE" JOURNAL OF IMMUNOLOGY, THE WILLIAMS AND WILKINS CO. BALTIMORE, US, vol. 157, 1996, pages 3216-3219, XP002952534 ISSN: 0022-1767 cited in the application page 3218, right-hand column, paragraph 2	1-10
Y	DOW STEVEN W ET AL: "Systemic and local interferon gamma gene delivery to the lungs for treatment of allergen-induced airway hyperresponsiveness in mice." HUMAN GENE THERAPY, vol. 10, no. 12, 10 August 1999 (1999-08-10), pages 1905-1914, XP002241814 ISSN: 1043-0342 cited in the application page 1908, right-hand column, paragraph 3	1-10
P,X	BEHERA ARUNA K ET AL: "Adenovirus-mediated interferon gamma gene therapy for allergic asthma: Involvement of interleukin 12 and STAT4 signaling." HUMAN GENE THERAPY, vol. 13, no. 14, 20 September 2002 (2002-09-20), pages 1697-1709, XP002241815 September 20, 2002 ISSN: 1043-0342 the whole document	1-10

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 03/06535

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-8 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Ir nal Application No
PCT/US 03/06535

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6218180 B1	17-04-2001	US 5952221 A	14-09-1999
		US 6531456 B1	11-03-2003
		CA 2247806 A1	12-09-1997
		EP 0885306 A2	23-12-1998
		JP 2002514900 T	21-05-2002
		WO 9732991 A2	12-09-199